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# Posttranscriptional Regulation of *Il10* Gene Expression Allows Natural Killer Cells to Express Immunoregulatory Function

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## SUMMARY

**Natural killer (NK) cells play a well-recognized role in early pathogen containment and in shaping acquired cell-mediated immunity. However, indirect evidence in humans and experimental models has suggested that NK cells also play negative regulatory roles during chronic disease. To formally test this hypothesis, we employed a well-defined experimental model of visceral leishmaniasis. Our data demonstrated that NKp46$^+$CD49b$^+$CD3$^-$ NK cells were recruited to the spleen and into hepatic granulomas, where they inhibited host protective immunity in an interleukin-10 (IL-10)-dependent manner. Although IL-10 mRNA could be detected in activated NK cells 24 hr after infection, the inhibitory function of NK cells was only acquired later during infection, coincident with increased IL-10 mRNA stability and an enhanced capacity to secrete IL-10 protein. Our data support a growing body of literature that implicates NK cells as negative regulators of cell-mediated immunity and suggest that NK cells, like CD4$^+$ T helper 1 cells, may acquire immunoregulatory functions as a consequence of extensive activation.**

## INTRODUCTION

Natural killer (NK) cells are known to provide a critical host defense system during the early phases of infection with a variety of viruses, fungi, bacteria, and parasites (Bancroft, 1993; Biron et al., 1999; Cerwenka and Lanier, 2001; Farag et al., 2002; Smyth et al., 2002; Trinchieri, 1989). NK cells do not possess conventional clonotypic antigen-specific receptors but are capable of spontaneously killing tumor cells and virus-infected cells that have downregulated one or more major histocompatibility complex (MHC) molecules and/or expressed certain stress antigens on their surface (Diefenbach and Raulet, 2003; Mehrotra et al., 1998). NK cells have also been shown to play an important role during pregnancy (Moffett-King, 2002), autoimmunity, and tissue inflammation (Homann et al., 2002; Shi et al., 2000).

Target recognition and cytokine stimulation are the two major triggering mechanisms for NK cells, and both shape their effector responses (Alli and Khar, 2004; Chakir et al., 2001; Fehniger et al., 1999; Lauwerys et al., 2000; Mehrotra et al., 1998). Considerable advances have been made in understanding the receptors that activate and inhibit functionally mature NK cells and in the cytokines leading to IFN-$\gamma$ production and cytoloytic activity. However, less is known about the regulation of other aspects of NK cell differentiation. In vitro studies have suggested that NK cells can also differentiate to produce interleukin-10 (IL-10) (Bodas et al., 2006; Grant et al., 2008; Moretta et al., 2002) and may have regulatory activity (Deniz et al., 2008). Although a negative regulatory role for NK cells mediated through IL-10 has been suggested during tumor development, in pregnancy, and most recently during persistent Hepatitis C virus infection (Barber et al., 2007; De Maria et al., 2007; Vigano et al., 2001), direct experimental evidence for a negative regulatory role of NK cells during infectious disease is lacking.

We have been investigating the underlying cellular events that regulate the effectiveness of host protective immunity after infection with the protozoan parasite *Leishmania donovani*. Resistance to this pathogen operates largely through the development of granulomatous inflammation, positively regulated by both T helper 1 (Th1) and Th2 cell cytokines (Stager et al., 2003) and negatively regulated largely by IL-10 (Murray et al., 2002). Although natural T regulatory (Treg) cells have received much attention in models of cutaneous leishmaniasis caused by *L. major* (Belkaid et al., 2002), in experimental visceral leishmaniasis (VL) caused by *L. donovani* (Stager et al., 2006), chronic cutaneous leishmaniasis caused by *L. major* Seidman (Anderson et al., 2007), and also during human kala azar (Nylen et al., 2007), the bulk of IL-10-producing CD4$^+$ T cells are inducible Foxp3$^-$ and CD25$^-$ regulatory T cells . A large fraction of these IL-10$^+$ CD4$^+$ T cells also coexpress IFN-$\gamma$, suggesting that they may, as in chronic toxoplasmosis (Jankovic et al., 2007), represent a further stage of Th1 cell differentiation (O'Garra and Vieira, 2007). We now show that after *L. donovani* infection, NK cells accumulate in the spleen and in hepatic granulomas of infected mice and that these NK cells represent another source of IL-10

during infection. Using adoptive transfer, we also provide formal evidence that NK cells from infected mice suppressed host resistance in an IL-10-dependent manner. Although upregulation of IL-10 gene expression was also found to be a feature of early NK cell activation, only NK cells isolated from mice with established infection could suppress host resistance, a function associated with the acquisition of increased IL-10 mRNA stability and an enhancement of IL-10 protein secretion. Thus, our data suggest a mechanism whereby prolonged in vivo activation gives rise to a population of NK cells with altered posttranscriptional regulation of IL-10 gene expression, heightened capacity for IL-10 protein production, and inhibitory function.

## RESULTS

### NK Cells Accumulate IL-10 mRNA during the Development of Experimental VL

IL-10 is a major contributor to disease progression in many diseases (Murphy et al., 2001; Murray et al., 2002; Sharma et al., 1999), and IL-10 production in experimental leishmaniasis has been attributed to $CD4^+$ T cells and macrophages (Anderson et al., 2007; Miles et al., 2005; Stager et al., 2006). As part of a more complete analysis of the IL-10 response to *L. donovani* infection, we examined IL-10 mRNA accumulation in a variety of highly enriched (>98% pure) cell populations in the absence of in vitro restimulation. In accord with our recent description of the late emergence of IL-10-producing $CD25^-Foxp3^-$ $CD4^+$ T cells (Stager et al., 2006), the accumulation of IL-10 mRNA within the splenic $CD4^+$ T cell population increased over time. However, we were surprised to note that $CD49b^+$ ($DX5^+$) NK cells, and not $CD4^+$ or $CD8^+$ T cells, B cells, $CD11c^{hi}$ cells, or macrophages, had accumulated most IL-10 mRNA by day 14 and that their mRNA accumulation equaled that of $CD4^+$ T cells at day 28 after infection (p.i.; Figure 1A). Thus, within the splenic populations examined here, NK cells represented a previously unrecognized potential source of IL-10 during this infection.

### NK Cells Expand in Number and Enter Hepatic Granulomas

During *L. donovani* infection, the spleen undergoes extensive architectural remodeling associated with splenomegaly, both characteristics also associated with human disease (Kaye et al., 2004). However, neither the number of NK cells nor their distribution has previously been reported. The latter has been problematic because of the lack of appropriate means to unambiguously identify NK cells in tissue sections in BALB/c mice. NKp46 has recently been described as a marker for NK cells in the mouse (Walzer et al., 2007) and is suitable for in situ detection of NK cells via immunohistochemistry. First, we confirmed expression of NKp46 on $CD49b^+$ splenic NK cells in naive BALB/c mice. As shown in Figure 1B (upper panel), more than 95% of $CD3^-CD49b^+$ cells stained brightly for NKp46. Using NKp46, we therefore determined the distribution of NK cells in the spleen of naive mice (Figure 1C). As suggested by previous studies (Andrews et al., 2001; Dokun et al., 2001; Walzer et al., 2007), $NKp46^+$ cells were located predominantly within the marginal zone and red pulp. As with $CD49b^+$ cells found in naive mice, almost all $CD49b^+$ NK cells in *L. donovani*-infected mice coexpressed NKp46 (Figure 1B, lower panel). In addition, of $CD3^-CD49b^+CD122^+$ cells, 95.5% ± 1.03% expressed NKp46 (compared to 97.4% ± 1.38% in naive mice; Figure S1 available online). In infected mice, a subset of $CD49b^+$ cells also expressed CD11c at low intensity, potentially allowing their misclassification as immature conventional (cDCs) or plasmacytoid DCs (Blasius et al., 2007; Caminschi et al., 2007; Vosshenrich et al., 2007). However, the $CD11c^{lo}CD49b^+$ cells observed in infected mice were uniformly $MHCII^-$ by flow cytometry, lacked intracellular MHCII by immunocytochemistry, did not express the costimulatory markers CD80 and CD86, and did not express either Gr-1 or B220 (Figures S2–S4 and data not shown). Staining with NKp46 therefore allowed us to unambiguously define NK cells in the spleen of mice infected with *L. donovani* (Figure 1D). NK cells were located within the red pulp and marginal zone, in a similar distribution to that of NK cells in naive mice.

In the liver of infected mice, immunity to *L. donovani* is expressed in granulomas (Engwerda and Kaye, 2000). To determine whether NK cells contributed to the cellular composition of hepatic granulomas, we first confirmed NKp46 expression on hepatic $CD49b^+CD3^-$ cells in both naive and infected mice (Figure 1E). Next, we performed immunohistochemistry with NKp46. The response of BALB/c mice has been well characterized previously and at day 14–28 p.i., granulomas can be seen in a variety of stages of maturation (Stager et al., 2003). $NKp46^+CD3^-$ cells were readily identified within immature and mature granulomas and also within the parenchyma (Figures 1F–1H). By thin-section analysis, ~75% of all detectable hepatic NK cells were within granulomas, and NK cells were observed in ~60% of the granulomas examined. Given the relatively low frequency of NK cells per granuloma, we cannot rule out the possibility that all granulomas might contain NK cells if examined across their entire volume.

To determine whether splenic and hepatic NK cells increased in number during infection, we quantified NK cells by flow cytometry. As shown in Figure 2A, in mice infected with *L. donovani*, the absolute number of splenic NK cells increased by 3-fold. A similar, though less pronounced, increase in hepatic NK cells was also observed (Figure 2B). To confirm the kinetics of acquisition of IL-10 mRNA by hepatic $CD49b^+NKp46^+$ NK cells in relation to $CD4^+$ and $CD8^+$ T cells, we again performed qRT-PCR on flow-sorted cells at different times after infection. As shown in Figures 2C and 2D, both splenic and hepatic NK cells with increased IL-10 mRNA accumulation were readily detected by day 7 p.i. Hepatic $CD4^+$ T cells with elevated levels of IL-10 mRNA appeared more rapidly than in spleen, whereas the kinetics of the IL-10 response in $CD8^+$ T cells was similar in both organs. Together, these data demonstrate that during the course of *L. donovani* infection, NK cells are one of the first populations to increase their potential for IL-10 production, that their numbers increase in infected tissues, and that they can home specifically into both the infected spleen and sites of granulomatous inflammation.

### IL-10-Producing NK Cells Suppress Host Resistance to *L. donovani*

Next, we wished to determine whether NK cells had the capacity to regulate the outcome of infection, as might be suggested by their increased accumulation of IL-10 mRNA. We first depleted NK cells in vivo by using anti-ASGM1. Treatment of mice with anti-ASGM1 has been commonly used to deplete NK cells,

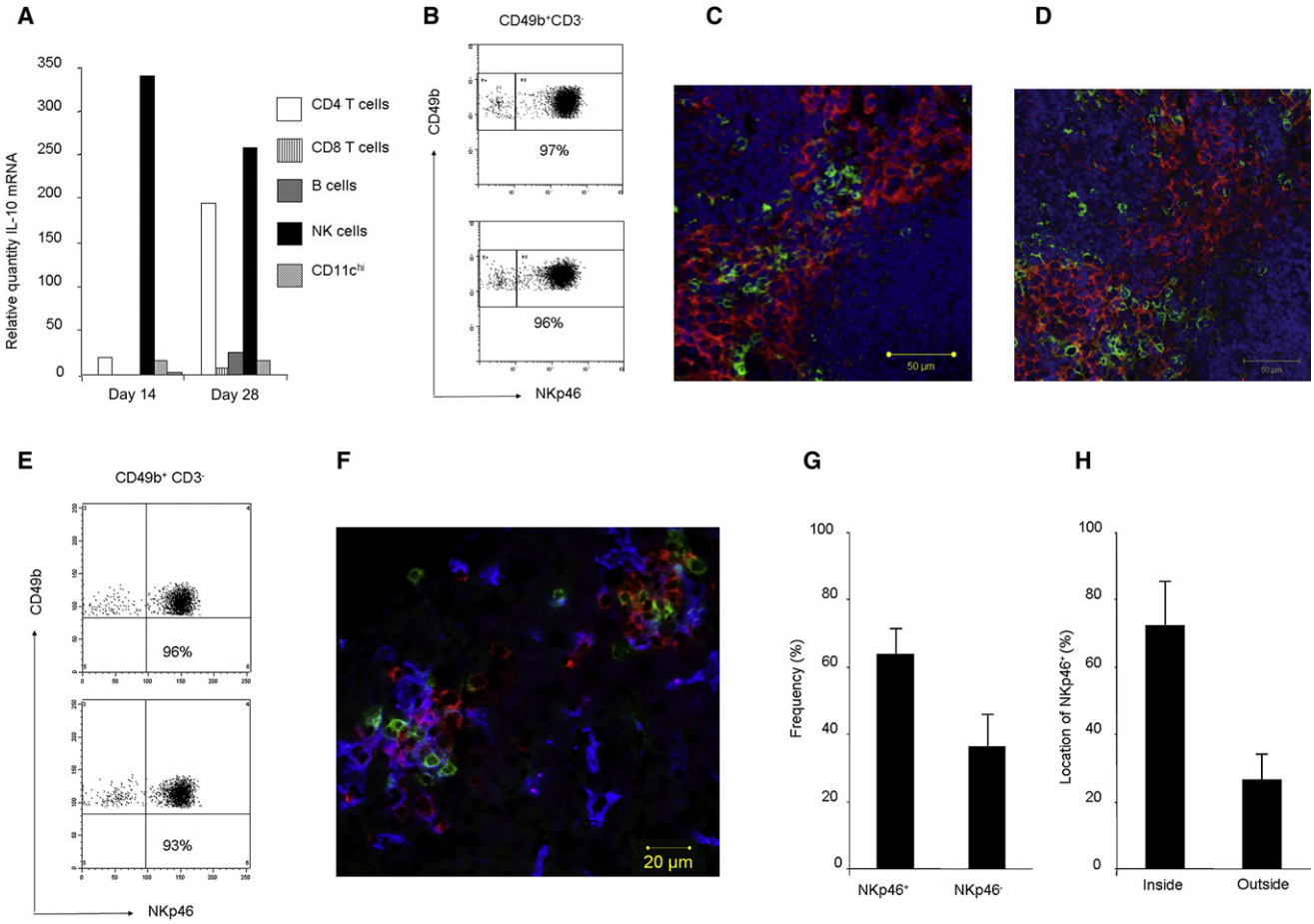


**Figure 1. Cellular Accumulation of IL-10 mRNA during *L. donovani* Infection**

(A) Spleen cell were prepared from naive BALB/c mice and BALB/c mice infected for 14 days or 28 days with *L. donovani*. $CD4^+CD3^+$ and $CD8^+CD3^+$ T cells, $B220^+$ B cells, $CD49b^+$ NK cells, $CD11c^{hi}$ DCs, and all residual cells (including macrophages and stromal cells) were sorted to > 99% purity and then examined for IL-10 mRNA accumulation by RT-PCR. Data represent mRNA samples from sorted cells isolated from pools of n = 5 spleens and are shown as n-fold difference over naive. One of three independent experiments is shown.

(B) Spleen cell suspensions from naive BALB/c mice (upper panel) and BALB/c mice infected for 28 days with *L. donovani* (lower panel) were stained for CD49b and NKp46. Plots are gated on $CD49b^+CD3^-$ cells.

(C and D) Immunohistology of (C) naive and (D) day 28-infected BALB/c spleen showing distribution of $NKp46^+$ NK cells (green) and $F4/80^+$ red-pulp macrophages (red). Sections were counterstained with DAPI (blue).

(E) Hepatic NK cells coexpress CD49b and NKp46. Cells from naive (upper panel) and day 28-infected (lower panel) BALB/c mice were gated as $CD49b^+CD3^-$. For further characterization, see Figure S1.

(F) $NKp46^+$ NK cells (green) colocalize with $F4/80^+$ macrophages (blue) and $CD3^+$ T cells (red) in hepatic granulomas caused by *L. donovani*.

(G and H) Frequency of granulomas identified as containing $NKp46^+$ cells (G) and the frequency of $NKp46^+$ cells located inside granulomas or outside granulomas, i.e., in the parenchyma (H), was enumerated from approximately 35–40 granulomas per mouse (in 20 random 8 μm sections). Data represent mean ± SD obtained from three mice.

though this antibody has known reactivity against other cells as well (Slifka et al., 2000). Antibodies were administered over the first 6 days of infection (see Experimental Procedures) and led to a rapid decrease in the frequency of $CD49b^+$ cells (from 2.27% in control-treated mice to 0.04% in mice treated for 24 hr with anti-ASGM1), which was sustained over the first 7 days p.i. Treatment with anti-ASGMI significantly reduced parasite burden in the spleen (to 55% ± 8.5% of control; $p < 0.001$) and liver (to 48% ± 9% of control; $p < 0.001$) of infected mice, as measured at day 28 p.i. Whereas ASGM1 stained < 2% of $CD4^+$ and < 5% of $CD8^+$ spleen cells in naive BALB/c mice, ASGM1 expression was markedly increased on T cells in infected mice (~15% and ~32% of $CD4^+$ and $CD8^+$ T cells, respectively, at day 21 p.i.; data not shown), making this approach unsuitable for analyzing the role of NK cells at later stages of infection. To circumvent this problem, we used an adoptive-transfer approach to test whether NK cells could influence the outcome of infection in otherwise unmanipulated hosts. Recipient BALB/c mice were infected with *L. donovani*, and at day 21 p.i. they were adoptively transferred with $10^6$ NK cells derived from either naive or day 21-infected mice. Parasite burden was then evaluated 7 days later (day 28 p.i.). At this time, hepatic resistance was beginning to be expressed, whereas parasite numbers were increasing in the spleen (Engwerda and Kaye, 2000). Transfer of

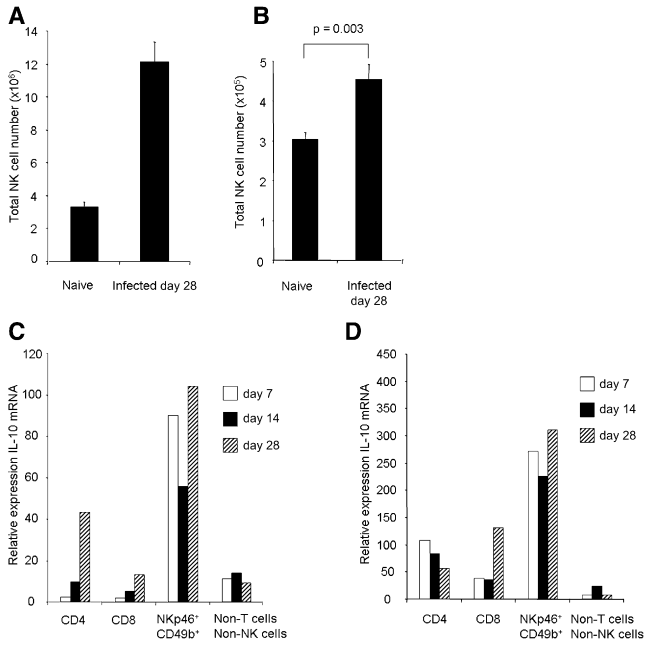


**Figure 2. Splenic and Hepatic IL-10$^+$ NK Cells Expand in Number during Experimental Visceral Leishmaniasis**

Total number (± SD) of splenic (A) and hepatic (B) CD3$^-$CD49b$^+$ NK cells in naive and 28 day-infected BALB/c mice (n = 4). Spleen (C) and liver (D) cells were prepared from naive BALB/c mice and BALB/c mice infected for 7 days, 14 days, or 28 days with *L. donovani*. CD4$^+$CD3$^+$ cells, CD8$^+$CD3$^+$ T cells, NKp46$^+$CD49b$^+$ cells, and all residual cells (including DCs, macrophages, and stromal cells) were sorted to > 99% purity and then examined for IL-10 mRNA accumulation by RT-PCR. Data represent mRNA samples from sorted cells isolated from pools of n = 5 spleens and are shown as n-fold difference over naive. Data from one of two independent experiments is shown.

NK cells isolated from the spleen of naive mice into animals that had been previously infected for 21 days had no effect on splenic (Figure 3A) or hepatic (Figure 3B) parasite burden. In contrast, adoptive transfer of splenic NK cells from day 21-infected mice into day 21-infected recipients significantly suppressed host resistance in both organs. In four independent experiments (n = 13–18 mice), NK cells from day 21-infected mice increased parasite burden by 2.47 ± 0.17-fold and 1.86 ± 0.16-fold in spleen and liver respectively ($p < 0.001$), whereas NK cells from naive mice increased parasite burden by 1.1 ± 0.16-fold and 1.25 ± 0.02-fold in spleen and liver, respectively (p = ns). These data indicated that NK cells from infected but not naive mice could inhibit host resistance to *L. donovani*. To confirm the fate of the adoptively transferred NK cells, we CFSE labeled NK cells from naive and infected mice prior to transfer, in order to allow visualization by immunohistochemistry and flow cytometry. Transferred NK cells could be detected in the spleen (Figures 3C and 3D), with a localization similar to that seen for endogenous NK cells (Figure 1D). By flow cytometry, the number of recovered NK cells was similar at 18 hr and 7 days after transfer (Figure 3E). These data suggest that the differences in suppressive function of NK cells from naive and infected mice were not due to differential survival or homing. Using this approach, we also confirmed that NK cells directly home into hepatic granulomas (Figures 3F and 3G). As with endogenous NK cells, transferred NK cells were observed in most, but not all, granulomas (Figure 3F).

As ~50% of NK cells in infected BALB/c mice expressed CD11c (Figure S2), we wished to determine whether this inhibitory function might be attributable to this emergent CD11c$^{lo}$ population of NK cells. However, as shown in Figures 3H and 3I, sorted CD11$^-$ and CD11c$^{lo}$ subsets of CD49b$^+$ NK cells equally inhibited host resistance (by 3.05 ± 0.5-fold and 3.41 ± 0.56-fold in the spleen and 2.59 ± 0.25-fold and 3.22 ± 0.24-fold in the liver for CD11c$^-$ and CD11$^{lo}$ NK cells, respectively; n = 5 and $p < 0.01$ in all cases versus no transfer or transfer of naive NK cells). In spite of this inhibitory activity, both CD11$^-$ and CD11c$^{lo}$ subsets also exhibited enhanced cytotoxic effector function compared to NK cells from naive mice (Figure S5). By these assays, diverse functions of NK cells did not, therefore, segregate on the basis of CD11c expression.

## *Il10*$^{+/+}$ but Not *Il10*$^{-/-}$ NK Cells Can Suppress Host Resistance to *L. donovani*

Although providing the first functional evidence that NK cells can suppress host resistance, these experiments did not directly address whether this was mediated via IL-10. To address this question, we developed a mixed-chimera approach, which allowed both progression of *L. donovani* infection and also the activation of both IL-10-sufficient and IL-10-deficient NK cells within the same infected host. We first generated mixed chimeras on the BALB background, transferring bone marrow derived from Thy1.2$^+$ BALB.*Il10*$^{-/-}$ and Thy1.1$^+$ BALB.*Il10*$^{+/+}$ into Thy1.1$^+$ BALB.*Il10*$^{+/+}$ recipients. However, as a result of limited expression of Thy1 on NK cells (Dunn and North, 1991; Rahal et al., 1991), it was not possible to recover sufficient *Il10*$^{-/-}$ and *Il10*$^{+/+}$ NK cells for subsequent adoptive transfer.

We therefore used CD45.1$^+$ B6.*Il10*$^{+/+}$ and CD45.2$^+$ B10.*Il10*$^{-/-}$ mice as an alternate genetic system. As in BALB/c mice, the number of CD3$^-$NKp46$^+$ NK cells in the spleen of infected B6 mice also increased during infection (from 2.7 ± 0.2 to 3.9 ± 1.5 × 10$^6$ per spleen), and these NK cells had increased accumulation of IL-10 mRNA compared to naive mice (8.4 ± 0.3-fold). However, the magnitude of the NK response in B6 mice, as measured by both these parameters, was clearly more muted than in BALB/c mice (Figures 1 and 2). Nevertheless, we generated (CD45.1$^+$ B6.*Il10*$^{+/+}$ + CD45.2$^+$ B10.*Il10*$^{-/-}$) → CD45.1 B6.*Il10*$^{+/+}$ irradiation chimeras and after 8 weeks of reconstitution, these mice were infected with *L. donovani* (Figure 4A). At day 28 p.i., IL-10-sufficient (CD49b$^+$CD45.1$^+$) and IL-10-deficient (CD49b$^+$ CD45.2$^+$) NK cells (Figure 4B) were present in similar ratios. Sorted (>98% purity) NK cells were then transferred into day 21-infected CD45.1$^+$ B6.*Il10*$^{+/+}$ recipient mice. Whereas IL-10-sufficent NK cells inhibited splenic (Figure 4C) and hepatic (Figure 4D) resistance to *L. donovani*, the transfer of *Il10*$^{-/-}$ NK cells had no effect compared to mice receiving no cell transfer. In two independent experiments, *Il10*$^{+/+}$ NK cells increased parasite burden by 2.58 ± 0.41-fold and 2.2 ± 0.29-fold in spleen and liver, respectively (n = 10, $p < 0.01$), whereas *Il10*$^{-/-}$ NK cells increased parasite burden by only 1.3 ± 0.22-fold and 1.12 ± 0.01-fold in spleen and liver, respectively (n = 10, ns). Even given the caveat that the NK cell response in B6 mice was less dramatic than that seen in BALB/c mice, these data provide a formal

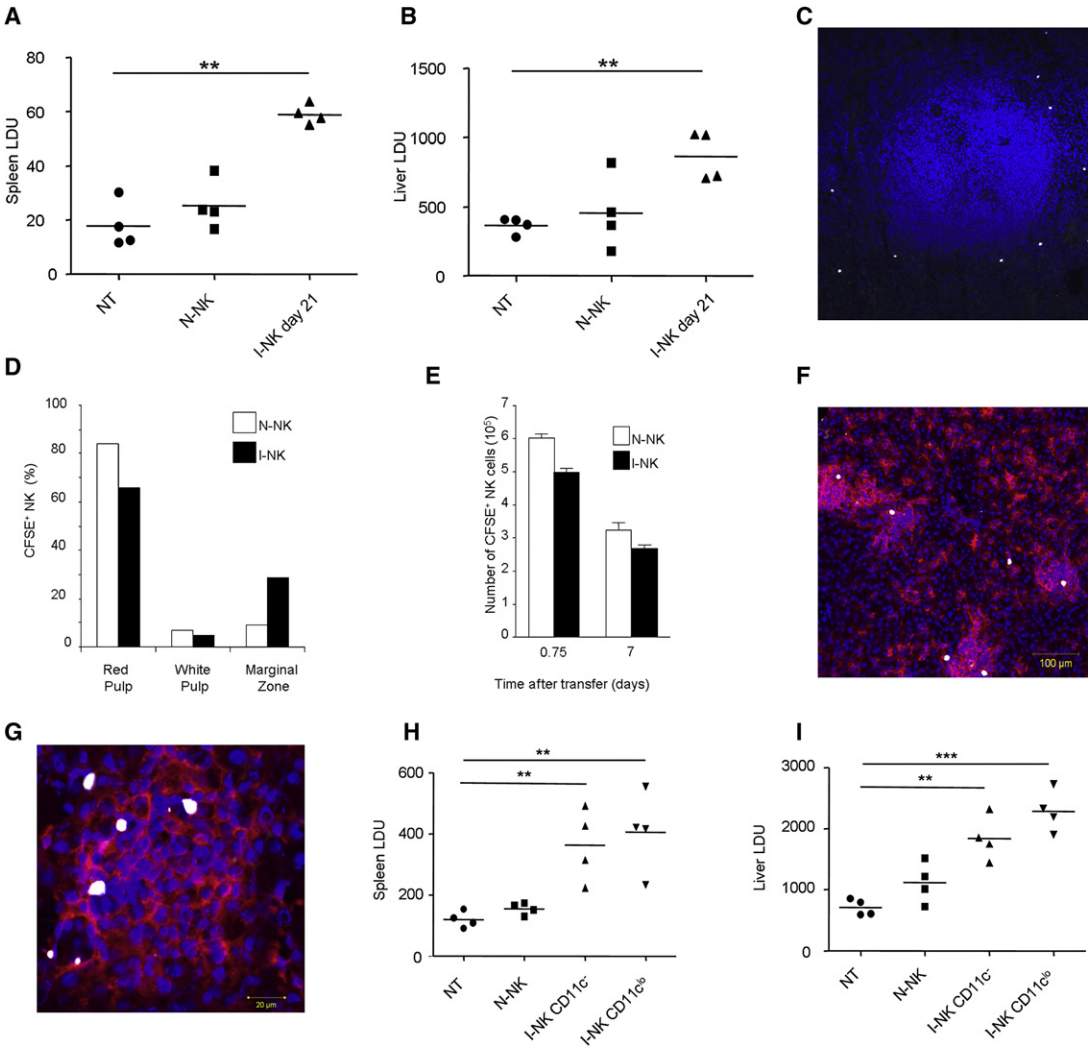


**Figure 3. Adoptive Transfer of NK Cells Demonstrates Their Capacity to Inhibit Host Resistance**

(A and B) NK cells isolated from naive BALB/c mice (N-NK) or day 21-infected BALB/c mice (I-NK) were flow sorted and transferred ($10^6$ i.v.) into day 21-infected BALB/c recipients. Control mice received no transfer (NT). (A) Splenic and (B) hepatic parasite burdens were determined 7 days after transfer. Symbols represent individual mice and bar represents mean value. Data from one of four independent experiments are shown.

(C) Immunohistology of day 21-infected BALB/c spleen showing distribution of transferred $CFSE^+$ NK cells (white). Sections were counterstained with DAPI (blue).

(D) Frequency of $CFSE^+$ NK cells distributed to the red pulp, white pulp, or marginal zone were enumerated 24 hr after transfer.

(E) CFSE-labeled NK cells ($2 \times 10^6$ i.v.) purified from naive and day 21-infected BALB/c mice were transferred into day 21-infected recipient mice; then, 18 hr and 7 days after transfer, donor NK cells were enumerated in the spleen (mean ± SD of n = 4 mice).

(F and G) Distribution of CFSE-labeled NK cells in liver granulomas caused by *L. donovani* with (F) low-magnification and (G) high-magnification images.

(H and I) NK cells from naive mice (N-NK) and from day 21-infected BALB/c mice (I-NK) sorted into their $CD11c^-$ and $CD11c^{lo}$ subsets were transferred into day 21-infected recipients, and 7 days later (H) splenic and (I) hepatic parasite burden was determined.

demonstration that the ability to produce IL-10 is essential for NK cells to suppress host resistance in vivo.

## Acquisition of an IL-10-Secreting Phenotype Requires Sustained NK Cell Activation

NK cells are activated within the first few days of *Leishmania* infection to secrete IFN-γ (Martin-Fontecha et al., 2004; Schleicher et al., 2007), but their ability to produce IL-10 early after infection has not been assessed. For further exploration of the kinetics of the IL-10 response, $CD49b^+$ NK cells were sorted from naive BALB/c mice and BALB/c mice infected for either 24 hr or 21 days with *L. donovani*. Unexpectedly, RT-PCR analysis indicated similar accumulation of IL-10 mRNA at both time points (Figure 5A). We then examined the capacity of NK cells isolated from 24 hr- and day 21-infected mice to inhibit host resistance. In contrast to NK cells isolated at day 21 p.i, NK cells isolated at 24 hr p.i. had no suppressive effect (Figures 5B and 5C). Thus, from three independent experiments (n = 10–14), NK cells from day 21- and from 24 hr-infected mice increased parasite burden by 2.47 ± 0.17-fold versus 1.01 ± 0.13-fold in the spleen ($p < 0.001$) and 2.16 ± 0.16-fold versus 1.05 ± 0.15-fold in the liver ($p < 0.001$). These data suggested that IL-10 mRNA

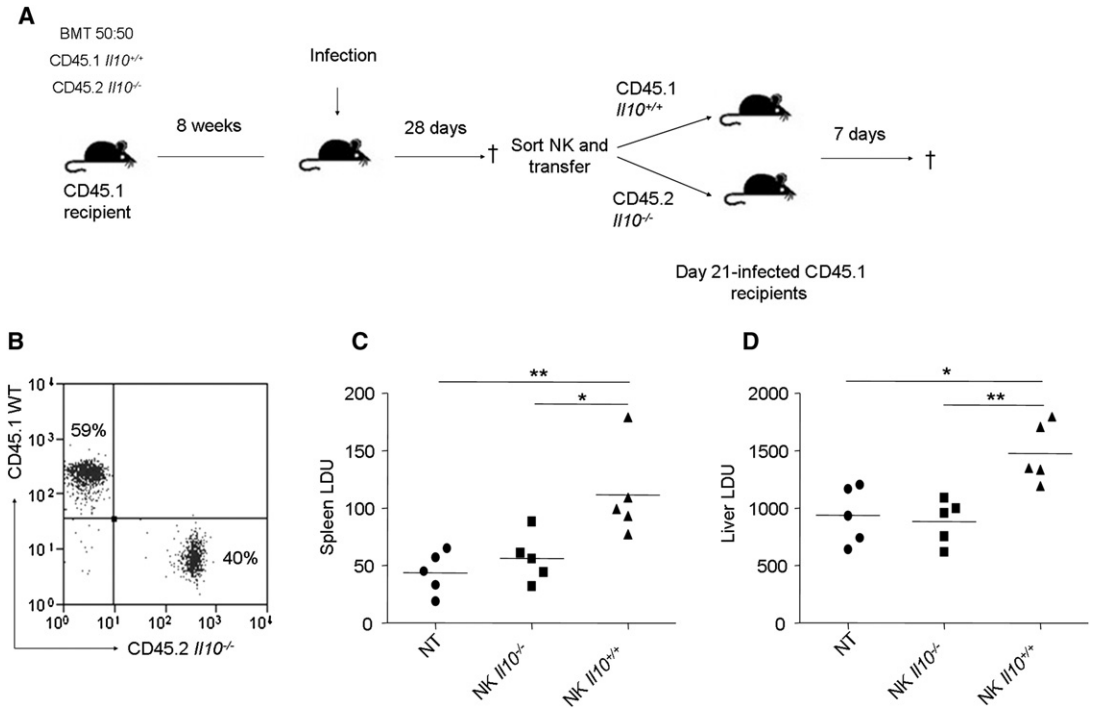


**Figure 4. Adoptive Transfer of NK Cells Demonstrates Their IL-10-Dependent Capacity to Inhibit Host Resistance**

(A) Schematic showing design for experiment with mixed chimeras.

(B) The frequencies of $CD49b^+$ IL-10-sufficient (CD45.1) and IL-10-deficient (CD45.2) splenic NK cells in infected chimeras was determined by flow cytometry. Plots are gated on $CD49b^+CD3^-$ cells.

(C and D) Sorted cells from day 28-infected chimeric mice were transferred into day 21-infected B6 mice, and 7 days later (C) splenic and (D) hepatic parasite burden was determined. Data from one of two independent experiments is shown. Symbols represent individual mice and the bar represents mean value.

accumulation could not reliably indicate the inhibitory function of NK cells in this assay. We therefore determined whether both populations of NK cells were similarly capable of secreting IL-10, as well as IFN-γ (Figures 5D–5F). NK cells were cultured in vitro in the presence or absence of rIL-12, because this cytokine has also been shown to induce both IL-10 and IFN-γ from NK cells (Mehrotra et al., 1998). NK cells from naive mice were readily activated in a dose-dependent manner by rIL-12 to produce IFN-γ (Figure 5D). In contrast, only low amounts of IL-10 (60–120 pg/ml) were produced at the highest dose of rIL-12 tested (5 ng/ml). NK cells from day 21-infected mice produced high amounts of IL-10 (500–1200 pg/ml), with even low doses of rIL-12 tested (Figure 5F). IL-10 secretion did not affect the production of IFN-γ, which was produced in similar concentrations and with similar kinetics to that found in cultures of NK cells from naive mice. In contrast, NK cells isolated from mice 24 hr p.i. had an enhanced IFN-γ response, yet produced minimal quantities of IL-10, requiring high doses of rIL-12 to elicit a response (Figure 5E). These data suggest that NK cells acquired the capacity to secrete high amounts of IL-10 as infection progressed.

Posttranscriptional regulation of IL-10 gene expression has been shown to control IL-10 secretion in a variety of cell types, including macrophages and keratinocytes (Powell et al., 2000). To explore whether this mechanism might account for the disparity between IL-10 mRNA accumulation and IL-10 protein secretion noted above, we used actinomycin D to block transcription and examine IL-10 mRNA stability. In NK cells isolated at 24 hr p.i., IL-10 mRNA decayed by almost 95% within 60 min of addition of actinomycin D (Figure 5G), similar to a recent report using cultured NK cells (Grant et al., 2008). In contrast, NK cells isolated at day 21 p.i. had retained ~60% of their initial IL-10 mRNA over a similar time period, representing an approximate doubling in relative mRNA half-life (~75 min versus ~30 min in NK cells from day 21- versus 24 hr-infected mice, respectively). These data suggest that altered mRNA stability contributes to the increased capacity of NK cells from day 21-infected mice to secrete IL-10. Together, our data indicate that over time, NK cells acquire the capacity to suppress hepatic resistance to *L. donovani* by a mechanism that involves posttranscriptional regulation of IL-10 gene expression, leading to elevated IL-10 protein production.

## DISCUSSION

NK cells play a vital role in early pathogen containment and by virtue of their capacity to produce IFN-γ, they are widely regarded as beneficial for host protection against intracellular pathogens. In this report, we demonstrate that NK cells have the capacity to act in the opposing manner, inhibiting host resistance to an intracellular pathogen through their production of IL-10.

Reports implicating NK cells as negative regulators of immunity are infrequent. Studies in IFN-γ-deficient mice have suggested that pulmonary NK cells contribute to the immunosuppressive environment in the lung after mycoplasma infection,

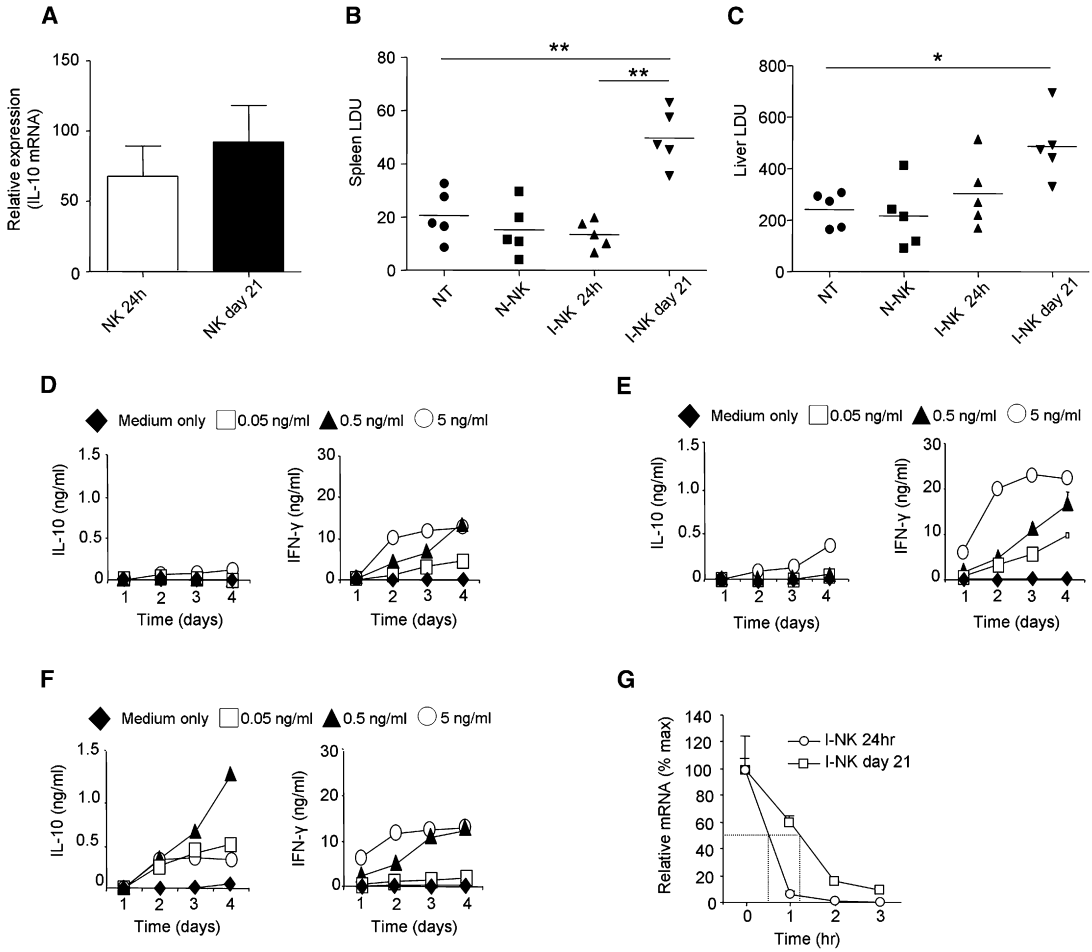


**Figure 5. Posttranscriptional Regulation of *Il10* Gene Expression and Increased IL-10 Secretion by NK Cells from *L. donovani*-Infected Mice**

(A) mRNA accumulation was determined in NK cells isolated from naive mice and mice infected for 24 hr or 21 days with *L. donovani*. Data are derived from four experiments (mean + SD), with mRNA obtained from NK cells pooled from 3–5 mice per experiment.

(B and C) NK cells from naive BALB/c mice (N-NK) and BALB/c mice infected for 24 hr or 21 days were transferred into day 21-infected BALB/c recipients, and (B) splenic and (C) hepatic parasite burden was determined 7 days later. Data represent one of three independent experiments.

(D–F) $CD49b^+$ NK cells were isolated from (D) naive BALB/c mice and BALB/c mice infected for (E) 24 hr or (F) 21 days with *L. donovani* and stimulated in vitro with increasing doses of rIL-12 (0.05–5 ng/ml) or cultured in medium alone. Supernatants were collected each day for 4 days, and IL-10 (left panels) and IFN-γ (right panels) was determined by ELISA.

(G) NK cells from mice infected for 24 hr and 21 days were cultured in the presence of actinomycin D. At indicated times, IL-10 mRNA accumulation was determined. Data are expressed as percentage of remaining IL-10 mRNA relative to mRNA levels at time 0 (168 versus 163 molecules of IL-10 mRNA per 1000 molecules HPRT in 24 hr and day 21 samples, respectively). Data represent one of two independent experiments.

and depletion of NK cells in IFN-γ-deficient animals prior to infection reduced the amount of IL-10 in BAL fluid (Woolard et al., 2005). NK cells can inhibit $CD4^+$ T cell IFN-γ production after murine cytomegalovirus infection (Su et al., 2001) and have been described to inhibit autoimmunity (Baxter and Smyth, 2002; Flodstrom et al., 2002; Horwitz et al., 1997), with a correlation between NK cell number and/or activity and periods of disease progression or remission being observed in multiple sclerosis and systemic lupus erythematosus (reviewed in (French and Yokoyama, 2004). Additionally, prior depletion of NK cells aggravates peptide-induced experimental allergic encephalomyelitis (Zhang et al., 1997). Uterine NK cells at the fetal-maternal interface are also thought to be essential at mediating tolerance to the fetus (Moffett-King, 2002). Nevertheless, NK cells are almost universally regarded as promoting antimicrobial immunity. Our finding that NK cells can inhibit host protection by production of IL-10 now dispels the notion that NK cells always operate to facilitate pathogen clearance.

IL-10 is well known as a critical regulator of immunity to *Leishmania* infection. IL-$10^{-/-}$ mice are highly resistant to *L. donovani* infection (Murphy et al., 2001; Murray et al., 2002), and IL-10 is essential for *L. major* persistence (Belkaid et al., 2002). Three sources of IL-10 have been examined in great detail. Miles et al. (2005) have highlighted the capacity of immune complexes to induce IL-10 production from macrophages, and, subsequently, a number of studies have indirectly shown a role for Ig in the establishment and maintenance of disease (Woelbing et al., 2006). Belkaid et al. (2002) established a critical role for IL-10 producing

$CD25^+Foxp3^+$ natural Treg cells in *L. major* infection. These cells act as a rheostat for the emerging $CD4^+$ Th1 response (Belkaid et al., 2002) and are vital for long-term memory to reinfection (Belkaid et al., 2002). Finally, $CD25^-Foxp3^-$ IL-10-producing $CD4^+$ T cells expand during both *L. donovani* (Stager et al., 2006) and *L. major* Seidman infection (Anderson et al., 2007). IL-10-producing $CD4^+$ T cells appear in greater numbers after day 21 of *L. donovani* infection in BALB/c mice, a process regulated by IL-6 produced by DCs (Stager et al., 2006). $CD25^-Foxp3^-$ $CD4^+$ T cells have also been identified as a source of IL-10 in humans infected with *L. donovani* (Nylen et al., 2007).

Here, we demonstrate that NK cells isolated from *L. donovani*-infected mice also produce IL-10 and confirm their ability to regulate disease outcome in vivo. Our data indicate that the well-defined early role for NK cells in enhancing Th1 differentiation (Cerwenka and Lanier, 2001) is not the full extent of NK cell function, and other immunoregulatory functions are acquired as infection progresses. To date, our analysis of mice after NK cell transfer has not revealed any significant alterations in the number of $CD4^+$ or $CD8^+$ T cells nor in the number or activation status of the major cDC subsets (data not shown). The ability to observe transferred NK cells with two-photon intravital microscopy (L.B., A.M., and P.M.K., unpublished data) and the availability of mice with cell-specific deficiency of IL-10R expression (R. Jack and W. Muller, unpublished data; Aidinis et al., 2008) should help elucidate their mechanism of action.

Although Nylen et al. (2007) did not identify NK cells as a major source of IL-10 mRNA in human disease, NK cells account for only ~3% of cells in splenic aspirate, and IL-10 secretion was not directly examined. Furthermore, the role of NK cells may well become overshadowed by that of $CD4^+$ T cells as disease progresses, as indeed is suggested by our data. Given that the presence of granulomatous inflammation has been associated with subclinical infection with *L. donovani* and that in the mouse model of VL, disease is less progressive than in humans (Kaye et al., 2004), our data demonstrating the modulation of parasite burden by NK cells suggest that IL-10-producing NK cells might have a more dominant role at early stages of human VL, perhaps contributing to the immunoregulatory balance that governs the transition from subclinical infection to clinical disease. Nevertheless, NK cell numbers do increase during fatal experimental visceral leishmaniasis in the hamster, a model often regarded as better reflecting end-stage human disease (Sartori et al., 1999).

Our data demonstrate that the capacity of NK cells to produce IL-10 protein is acquired over the course of infection, whereas the capacity to produce IFN-$\gamma$ remains largely unaltered. The precise molecular mechanism(s) involved remain to be defined, though our data point to enhanced IL-10 mRNA stability as a contributing factor. Although posttranscriptional regulation of cytokine genes in NK cells has not been described, a precedent for the control of IL-10 secretion by such a mechanism is found in macrophages, where IL-10 mRNA stability has been shown to be associated with regulatory elements in the 3′ UTR (Powell et al., 2000). Further studies will be required to determine whether analogous mechanisms exist in murine NK cells. Studies carried out by Brady et al. (2004) demonstrated that stimulating NK cells with IL-2 and IL-21 can induce IL-10 production. We show here that IL-12 can induce NK cells from infected mice to secrete IL-10, and human NK cells respond to IL-2 and IL-12 with IL-10 secretion (Moretta et al., 2002). Furthermore, recent studies indicate that in cultured murine NK cells, these cytokines act synergistically to drive Stat4-dependent but T-bet-independent induction of IL-10 mRNA (Grant et al., 2008). Thus, it is tempting to speculate that IL-2 and IL-12 may also play a role in the development and/or maintenance of IL-10-producing NK cells during experimental leishmaniasis. In support of such an association, we have previously shown that the frequency of $IL\text{-}12p40^+$ cells in hepatic granulomas increases significantly between day 14 and day 28 p.i. (Engwerda et al., 1998), mirroring the kinetics of emergence of IL-10-producing NK cells described here. IL-2 has also been linked to the generation of IL-10 during *L. donovani* infection (Bodas et al., 2006). Whether these cytokines, or other environmental factors, are ultimately responsible for modulating NK cell function remains to be established, however.

Finally, our data suggest striking, if not mechanistic, parallels in the functional differentiation of NK cells and $CD4^+$ Th1 cells. Recent studies (Jankovic et al., 2007) have identified conventional IFN-$\gamma$-producing Th1 cells as a major source of IL-10 after *Toxoplasma gondii* infection, proposing that expression of IL-10 helps to suppress APC function as a regulatory mechanism to limit immunopathology. Our data suggest that NK cells, under the influence of an evolving inflammatory response, likewise further differentiate from a population characterized by cytolytic effector activity and IFN-$\gamma$ production, into one that also has the ability to secrete IL-10. Identifying the factors responsible for promoting this switch in cytokine profile may open new avenues for therapeutic regulation of NK cell activity in a variety of chronic infectious and noninfectious inflammatory states.

## EXPERIMENTAL PROCEDURES

### Mice and Infection

BALB/c and C57BL6 mice were obtained from Charles River (UK), B10.*Il10*$^{-/-}$ mice were originally developed by DNAX and were obtained from A. Sher (NIAID, NIH), and BALB.Thy1.1 and BALB.*Il10*$^{-/-}$ (Thy1.2) and hCD2-GFP (de Boer et al., 2003) mice were the kind gift of J. Langhorne, A. O'Garra, and D. Kioussis, respectively (NIMR, London). Mice were housed under specific pathogen-free conditions and used at 6–8 weeks of age. The Ethiopian strain of *Leishmania donovani* (LV9) was maintained by serial passage in Syrian hamsters. Amastigotes were isolated from infected spleens, as previously described (Stager et al., 2000), and mice were infected with $2 \times 10^7$ *L. donovani* amastigotes intravenously (i.v.) via the tail vein in 200 μl of RPMI 1640 (GIBCO, Paisley, UK). Mice were sacrificed by cervical dislocation, and parasite burdens in the liver and the spleen were determined from Giemsa-stained impression smears (Engwerda et al., 1998). Parasite burden was expressed as Leishman-Donovan units (LDUs; i.e., the number of parasites per 1000 host cell nuclei × organ weight) (Smelt et al., 1997). All experiments were approved by the London School of Hygiene and Tropical Medicine (LSHTM) and University of York Animal Procedures and Ethics Committee and performed under UK Home Office license.

For the generation of chimeras, donor mice were irradiated twice with 1100 rads on a split-dose regimen (550 rad on day 1 and day 2) and then were reconstituted i.v. via the tail vein on day 2 with $2 \times 10^6$ T cell-depleted bone marrow cells per mouse, comprising a 1:1 ratio of *Il10*$^{+/+}$ and *Il10* $^{-/-}$ cells (see text for details). At 6–8 weeks after reconstitution, mice were examined for chimerism by flow cytometry and infected as described above. For adoptive transfer, sorted splenic NK cell subsets from either naive or infected mice were injected ($1 \times 10^6$/mouse) i.v. into day 21-infected mice, and 1 week later splenic and hepatic parasite loads were determined.

### Splenic DC and NK Enrichment

Splenic NK cells from naive and infected mice were enriched by digesting the spleens in RPMI supplemented with 0.1 mg/ml collagenase for 25 min at room temperature (RT). $B220^+$, $CD19^+$, $CD3\varepsilon^+$, and highly phagocytic cells were separated with magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and a MidiMACS separation column (Miltenyi Biotec). $CD3^-CD49b^+$ NK cells were then purified with either a FACS Vantage (BD Biosciences, Mountain View, CA) or a MoFlo (Dakocytomation) cell sorter. Sort gates are described in the Results. Sorted cells exceeded > 98% purity.

### Flow Cytometry

For flow cytometry, cells were incubated with 10 μg/ml 2.4G2 anti-FcRγII/III mAb (ATCC, Rockville, MD) followed by staining with directly conjugated monoclonal antibodies. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated MHCII (2G9) and CD3 (145-2C11) antibodies; phycoerythrin (PE)-conjugated CD45RB (16A), CD44 (IM7), Ly49b (DX5), NK 1.1 (PK136), and Gr-1 (RB6-8C5) antibodies; biotinylated CD11c (HL3), MHC-II (2G9), CD4 (RM4-5), CD8 (53-6.7), CD80 (16.10A1), CD86 (GL1), CD19 (1D3), B220/CD45R (RA3-6B2), and CD3e (145-2C11) antibodies; and APC-conjugated CD11c (clone HL3) antibody (all from BD PharMingen, San Diego, CA). Biotinylated antibodies were visualized with PerCP-streptavidin (BD PharMingen). NKp46 expression was detected by use of a polyclonal antibody against mouse NKp46 (R&D systems). Secondary staining was carried out with a donkey anti-goat IgG alexa 488 antibody (Invitrogen). Some samples were also stained with rabbit anti-ASGM1 (Cedarlane, Ontario, Canada) followed by anti-rabbit alexa 647 (molecular probes). Minimal background staining was observed with appropriate control FITC-conjugated, PE-conjugated, biotinylated, and APC antibodies. All staining was performed on ice in phosphate-buffered saline (PBS) containing 2% FCS and 5 mM EDTA for 30 min. Flow-cytometric analysis was performed with a FACSCalibur (BD Biosciences, Mountain View, CA) or a Cyan (Dakocytomation). Fifty thousand cells were acquired and analyzed with either CellQuest (BD Biosciences) or Summit (Dakocytomation) software. Samples were spiked with a known concentration of microbeads (Polysciences) prior to acquisition in order to obtain absolute numbers of cells.

### In Vivo Depletion of $ASGM1^+$ Cells

BALB/c mice were injected with 20 μl of rabbit polyclonal anti-ASGM1 (Cedarlane) or control polyclonal normal rabbit IgG (Sigma) in a total volume of 200 μl/mouse 24 hr prior to infection and days 2, 4, and 6 p.i. Liver LDUs were determined at day 28 after infection.

### Cytotoxicity Assay

YAC-1 cells ($10^3$/well) were used as targets for NK cell-mediated lysis. In brief, NK cell subsets isolated from naive and day 28-infected animals were seeded in 96-well round-bottom plates at different E/T ratios and incubated at 37°C for 4 hr. Each test sample was plated in triplicate. Cytotoxicity was measured with the LIVE/DEAD Viability/Cytotoxity kit (Invitrogen) according to manufacturer's instructions and published protocols (Papadopoulos et al., 1994).

### Real-Time RT-PCR

RNA was isolated from sorted cell subsets with an RNeasy kit, according to the manufacturer's instructions (QIAGEN). RNA was reverse transcribed into cDNA with the first-strand cDNA synthesis kit according to the manufacturer's instructions (Invitrogen). Oligonuclotides (5′–3′) used for the specific amplification of *Il10* and *Hprt* were as described in (Ato et al., 2002). The real-time quantitative PCR was performed with the SYBR Green PCR kit in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to manufacturer's instructions. Accumulation of *Il10* was normalized to *Hprt* and expressed as either absolute copy number (target molecules/1000 *Hprt* molecules) or relative expression via the change in cycle threshold (ΔΔCT) analysis method (relative expression in infected versus naive mice).

### Immunofluorescence

For immunofluorescence, cytospun cells were fixed in 4% paraformaldehyde for 30 min at RT. After fixing, samples were quenched with 50 mM $NH_4Cl$ in PBS for 30 min at RT and then blocked and permeabilized by incubation with 1.5% v/v normal goat serum in 0.1% v/v saponin in PBS for 45 min at RT. Samples were incubated with biotin-conjugated rat anti-MHC-II (clone 2G9, BD PharMingen) and hamster anti-CD11c (clone N418, Serotec) diluted in 0.1% v/v saponin in PBS and incubated for 45 min at RT. After washing, specific staining was detected by Alexa 488-conjugated streptavidin and Alexa 546-conjugated goat-anti hamster IgG (Invitrogen). Slides were washed three times with 0.1% v/v saponin in PBS, and then coverslips were mounted in Prolong Gold (Invitrogen) and visualized with a 63× (NA 1.4) Plan-Apochromat oil-immersion objective with a Zeiss Axioplan LSM 510 confocal microscope.

### Confocal Microscopy

Confocal was performed on 8-μM-thick serial frozen sections. Sections were fixed with ice-cold acetone before staining with anti-CD3 PE, anti-F4/80 biotin, and goat anti-mouse NKp46 followed by secondary donkey anti-goat IgG alexa 488 (molecular probes) and streptavidin 546. DAPI was used as a nuclear counterstain. Slides were analyzed by confocal microscopy (Zeiss LSM 510).

### mRNA Stability Determination

Purified NK cells isolated from infected BALB/c mice at 24 hr and day 21 p.i. were incubated in the presence of actinomycin D (5 μg/ml). The cells were collected for RNA extraction at 60 min intervals for 3 h after the addition of actinomycin D, and data are plotted as percentage of *Il10* mRNA remaining relative to that determined at the addition of actinomycin D.

### ELISA

Purified $CD3^-CD49b^+$ NK cells were cultured ($1 \times 10^5$ cells / well) in various doses of IL-12. Supernatants were harvested at 24 hr intervals for 4 days. IL-10 and IFN-γ levels in supernatants were assayed with a sandwich ELISA kit (BioSource) according to the manufacturer's instruction.

### Statistics

Statistical analysis was performed with a paired Student's t test or a Mann Whitney U test, as appropriate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

## SUPPLEMENTAL DATA

Supplemental Data include five figures and can be found with this article online at http://www.immunity.com/cgi/content/full/29/2/295/DC1/.

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